Analysis of Hair Samples for Sympathomimetic Amines

1 Introduction

Sympathomimetic amines (SMAs) are generally a class of synthetic phenethylamine-derived drugs often generically referred to as "amphetamines". Almost all of these compounds show some degree of stimulant effects, but a wide variety of additional structure-dependent pharmacological effects can be seen in various compounds. These include pure stimulants (amphetamine and methamphetamine), decongestants (phenylpropanolamine and pseudoephedrine), anorexics (phentermine and fenfluramine), and hallucinogens (mescaline, one of the few relevant naturally occurring SMAs). Over the last few decades there has been particular interest in and concern over the widespread illicit use of various "designer" SMAs with combined stimulant and hallucinogenic properties. The "type specimen" of this class is 3,4-methylenedioxy-methamphetamine (MDMA or "ecstasy"), which was originally developed for possible use as an adjunct drug in psychotherapy, but now one of the most widely used illicit drugs in teenage and young adult populations. Chemists in clandestine drug laboratories have developed a wide array of related compounds, including thioalkyl- and halogen-containing analogues, in attempts to stay ahead of drug scheduling regulations.

2 Scope

This procedure allows for screening, confirmation and quantitation of the following SMAs in hair samples: amphetamine, methamphetamine, ephedrine / pseudoephedrine, methylenedioxyethylamphetamine (MDEA), and methylenedioxymethamphetamine (MDMA). It also allows for the qualitative analysis of hair samples for methylenedioxyamphetamine (MDA).

3 Principle

Hair samples are quantitatively assayed for SMAs. After washing, specimens are dried and cryoground into a powder. The resulting hair powder is mixed with an internal standard (normally a mixture of six deuterated SMAs) and extracted in methanol overnight. The methanol extracts are then taken to dryness and reconstituted in water. The aqueous extracts are adjusted to a basic pH, and extracted with hexane. The hexane is removed, acidified to prevent evaporation of volatile SMAs, and taken to dryness. The resulting residue is reconstituted in 10/90 methanol/water and analyzed by liquid chromatography with high resolution mass spectrometry (LC-FTMS). The extraction procedure is derived from work by Sadeghipour and Veuthey. The chromatographic and mass spectral procedures and parameters were developed in-house.

4 Specimens

This procedure uses 50 mg of hair if specimens are analyzed in duplicate.

5 Equipment/Materials/Reagents

Guidance for the preparation of reagents may be found in the *Preparation of Chemical Reagents* standard operating procedure (Tox 103).

a. 16x100 mm screw-top tubes with Teflon-lined caps

b. 12x75 mm culture tubes with polypropylene snap-tops

c. Acetonitrile (Optima grade or better)

d. Formic Acid (Puriss grade or better)

e. Hexane (UV grade or better)

f. Hydrochloric acid (ACS grade or better)

g. Methanol (Optima grade or better)

h. Sodium hydroxide (ACS grade or better)

i. Water (Deionized and Optima or better grade)

j. 4% Sodium hydroxide
Dissolve 2 g sodium hydroxide in 50 mL deionized water. Store in plastic at room temperature. Stable for at least 6 months.

k. Methanol:Hydrochloric Acid (4:1 v:v)
Mix 20 mL methanol with 5 mL hydrochloric acid. Store in glass at room temperature. Stable for at least 1 month.

l. Methanol:Water (10:90 v:v)
Mix 5 mL methanol with 45 mL water (both Optima grade or better). Store in glass at room temperature. Stable for at least 1 year.

m. 0.1% Formic acid in acetonitrile
Vacuum filter 500 mL acetonitrile through a 5 µm PTFE membrane and mix with 0.5 mL formic acid. Store in glass at room temperature. Stable for 2 months.

n. 0.1% Formic acid in water
Vacuum filter 500 mL water (Optima grade or better) through a 5 µm PTFE membrane and mix with 0.5 mL formic acid. Store in glass at room temperature. Stable for 2 months.

o. Vortex mixer, Rotator, Centrifuge, Heating Block and Cryogrinder

p. Evaporator with nitrogen

q. Routine laboratory supplies, including disposable pipettes, wooden sticks, test tube racks, graduated cylinders, etc.

r. Liquid Chromatograph-Orbitrap Mass Spectrometer

s. HPLC Column (Alltech Alltima C18, 2.1 x 150 mm, 5 µm dp, with a 2.1 x 7.5 mm guard column; or equivalent)

t. Methylene chloride (HPLC grade)

u. Disposable magnetic stir bars

v. Ultrafree-CL centrifuge filters (0.45 µm PVDF)

6 Standards and Controls

a. d_3-Ephedrine Stock Solution (100 µg/mL):
A methanol solution purchased from Cerilliant or other approved vendor. Stability and storage conditions are determined by the manufacturer.

b. d_5-Amphetamine Stock Solution (100 µg/mL):
A methanol solution purchased from Cerilliant or other approved vendor. Stability and storage conditions are determined by the manufacturer.

c. d_5-Methamphetamine Stock Solution (100 µg/mL):
A methanol solution purchased from Cerilliant or other approved vendor. Stability and storage conditions are determined by the manufacturer.

d. d_5-MDA Stock Solution (100 µg/mL):
A methanol solution purchased from Cerilliant or other approved vendor. Stability and storage conditions are determined by the manufacturer.

e. d_5-MDMA Stock Solution (100 µg/mL):
A methanol solution purchased from Cerilliant or other approved vendor. Stability and storage conditions are determined by the manufacturer.

f. d_5-MDEA Stock Solution (100 µg/mL):
A methanol solution purchased from Cerilliant or other approved vendor. Stability and storage conditions are determined by the manufacturer.

g. Internal Standard Working Solution (2 µg/mL of each component):
Combine 0.5 mL each of the d_3-ephedrine, d_5-amphetamine, d_5-methamphetamine, d_5-MDA, d_5-MDMA, and d_5-MDEA stock solutions in a 25 mL volumetric flask. Add 2 mL methanol and bring to the mark with water (both Optima grade or better). Store in glass at <0ºC. Stable for at least 2 years.

h. Hair Internal Standard Working Solution (25 ng/mL of each component):
Add 625 µL of the Internal Standard Working Solution (2 µg/mL) to a 50 mL volumetric flask and bring to the mark with Optima grade methanol. Store in glass at <0ºC. Stable for at least 2 years.

i. Ephedrine Stock Solution (1 mg/mL):
A methanol solution purchased from Cerilliant or other approved vendor. Stability and storage conditions are determined by the manufacturer.

j. MBDB (N-methylbenzodioxazolylbutanamine, N-methyl-1-(3,4-methylenedioxy-phenyl)-2-butanamine) Stock Solution (1 mg/mL):
A methanol solution purchased from Cerilliant or other approved vendor. Stability and storage conditions are determined by the manufacturer.

k. Amine Mixture-6 (250 µg/mL each component):
A methanol solution containing amphetamine, methamphetamine, phentermine, MDA, MDMA, and MDEA purchased from Cerilliant or another approved vendor. Stability and storage conditions are determined by the manufacturer.

l. Column Performance Evaluation Mix (1 µg/mL each component):
Combine 25 µL each of the MBDB and ephedrine stock solutions with 100 µL of the Amine Mixture-6 in a 25 mL volumetric flask. Add 2.4 mL methanol and bring to the mark with water (both Optima grade or better). Stable for at least 2 years. A 10 µL portion of

this solution is analyzed before each day's samples, in order to confirm acceptable instrument performance.

m. Amphetamine Stock Solution (1 mg/mL):
A methanol solution purchased from Cerilliant or other approved vendor. Stability and storage conditions are determined by the manufacturer.

n. Methamphetamine Stock Solution (1 mg/mL):
A methanol solution purchased from Cerilliant or other approved vendor. Stability and storage conditions are determined by the manufacturer.

o. MDA Stock Solution (1 mg/mL):
A methanol solution purchased from Cerilliant or other approved vendor. Stability and storage conditions are determined by the manufacturer.

p. MDMA Stock Solution (1 mg/mL):
A methanol solution purchased from Cerilliant or other approved vendor. Stability and storage conditions are determined by the manufacturer.

q. MDEA Stock Solution (1 mg/mL):
A methanol solution purchased from Cerilliant or other approved vendor. Stability and storage conditions are determined by the manufacturer.

r. Control Working Solution (1 µg/mL each component):
Mix 50 µL each of the ephedrine, amphetamine, methamphetamine, MDA, MDMA, and MDEA stock solutions in a 50 mL volumetric flask. Add 7 mL methanol and bring to the mark with water (both Optima grade or better). Store in glass at <0ºC. Stable for at least 1 year.

s. Hair Control Working Solution (125 ng/mL each component):
Add 1.25 mL of the Control Working Solution (1 µg/mL) to a 10 mL volumetric flask and bring to the mark with Optima grade methanol. Store in glass at <0ºC. Stable for at least 1 year.

t. Calibration Working Solution #1 (5 µg/mL each component):
Mix 250 µL each of the ephedrine, amphetamine, methamphetamine, MDA, MDMA, and MDEA stock solutions in a 50 mL volumetric flask. Add 8.5 mL methanol and bring to the mark with water (both Optima grade or better). Store in glass at <0ºC. Stable for at least 1 year.

u. Calibration Working Solution #2 (0.5 µg/mL each component):
Mix 25 µL each of the ephedrine, amphetamine, methamphetamine, MDA, MDMA, and MDEA stock solutions in a 50 mL volumetric flask. Add 9.9 mL methanol and bring to the mark with water (both Optima grade or better). Store in glass at <0ºC. Stable for at least 1 year.

v. Hair Calibration Working Solution #3 (125 ng/mL each component):
Add 625 µL of the Calibration Working Solution #1 (5 µg/mL) to a 25 mL volumetric flask and bring to the mark with Optima grade methanol. Store in glass at <0ºC. Stable for at least 1 year.

w. Hair Calibration Working Solution #4 (12.5 ng/mL each component):
Add 625 µL of the Calibration Working Solution #2 (0.5 µg/mL) to a 25 mL volumetric flask and bring to the mark with Optima grade methanol. Store in glass at <0ºC. Stable for at least 1 year.

x. Negative Control Hair:
Purchased from Diagnostics Products Corporation, UTAK Laboratories, Inc., Cliniqa, or prepared in-house from an appropriate blank specimen. Hair will be stored at room temperature, and does not expire. A Negative Control hair sample will be extracted and analyzed with every assay.

y. Positive Control Hair:
This is normally prepared in-house as per the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101), but may be purchased from an appropriate vendor as needed. When prepared in house, it will be made fresh. Normally prepared at concentrations of 50 and 500 pg/mg by adding 10 and 100 µL of the Hair Control Working Solution to 25 mg of Negative Control Hair. Other levels may be used as circumstances dictate.

7 Calibration

This procedure may be used quantitatively via construction of a multi-point calibration curve for the analyte(s) of interest following the *Guideline for Toxicological Quantitations* standard operating procedure (Tox 101). Table 1 shows typical calibrators and preparation instructions for hair calibrators.

Table 1: Hair Calibrator Preparation

Cal Level (pg/mg)	Hair Amount (mg)	Hair Calibrator Working Solution #3 Volume (µL)	Hair Calibrator Working Solution #4 Volume (µL)
25	25	●	5●
5●	25	●	1●●
75	25	●	15●
175	25	35	●
3●●	25	6●	●
5●●	25	1●●	●
75●	25	15●	●
1●●●	25	2●●	●

8 Sampling

Not applicable.

9 Procedure

Appendix 1 contains an abbreviated version of this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

Preparation of Hair Samples:

a. Visually inspect hair and record observations.

b. If segmental analysis is required, cut a portion of the hair sample into 2-cm segments.

c. Accurately weigh 25-1●● mg of each hair sample into a properly labeled test tube (to the nearest ●.1 mg).

d. Wash each hair sample with 1.5 mL methanol by vortexing for approximately 1 minute. Discard this wash.

e. Wash each hair sample with 1.5 mL methylene chloride by vortexing the sample for approximately 1 minute. Discard this wash.

f. Wash each hair sample with 1.5 mL methanol by vortexing for approximately 1 minute. Save this final wash for later analysis, if necessary. Control washes need not be saved.

g. Dry hair samples in a heating block at approximately 40°C to evaporate any remaining solvent.

h. Cryogrind dry hair samples in the freezer mill using the settings in Section 10.3 of this procedure.

i. Accurately weigh 25 mg of hair powder to a small vial (to the nearest 0.1 mg). Samples will be prepared in duplicate if specimen size allows. Smaller amounts may be weighed to account for high concentrations of analyte and/or limited specimen amount.

j. Add a magnetic stir bar, 1.5 mL methanol, and 50 µL Hair Internal Standard Working Solution (25 ng/mL) to each vial.

k. Extract overnight (at least 12 hours) with stirring at 37°C.

l. Filter the methanol extract using an Ultrafree-CL 0.45 µm centrifuge filter by spinning at 3000 rpm for 5 minutes. Discard the stir bar and hair.

m. Add 0.1 mL of 4:1 methanol:hydrochloric acid to filtrate and vortex briefly.

n. Evaporate to dryness under a gentle stream of nitrogen at approximately 40ºC.

o. Reconstitute each sample in 0.5 mL deionized water by vortexing for at least 10 seconds.

For Hair Extracts:

a. To a properly labeled 16x100 mm screw-top tube add 0.5 mL of water based hair extract.

b. Add 0.2 mL of 4% sodium hydroxide to each sample and vortex briefly.

c. Add 2 mL of hexane to each tube and extract for 20 minutes on a rotator. Centrifuge 10 minutes at a minimum of 3000 rpm. Use a wooden stick to break up any emulsions that develop.

d. Transfer organic (top) layer to a 12x75 mm culture tube.

e. Add 0.1 mL of 4:1 methanol:hydrochloric acid and vortex briefly.

f. Evaporate the hexane to dryness under a gentle stream of nitrogen at approximately 40ºC.

g. Reconstitute the dried residue in 0.1 mL of 10:90 methanol:water.

h. Analyze by LC-FTMS using the conditions given below (Sections 10.1 and 10.2).

For Wash Samples:

a. For samples in which an SMA is identified above the LLOQ of the method, add 25 µL Hair Internal Standard Working Solution (25 ng/mL) to each wash.

b. Add 0.1 mL of 4:1 methanol:hydrochloric acid and vortex briefly.

c. Evaporate to dryness under a gentle stream of nitrogen at approximately 40ºC.

d. Reconstitute each sample in 0.5 mL deionized water by vortexing for at least 10 seconds.

e. Add 0.2 mL of 4% sodium hydroxide to each sample and vortex briefly.

f. Add 2 mL of hexane to each tube and extract for 20 minutes on a rotator. Centrifuge 10 minutes at a minimum of 3000 rpm. Use a wooden stick to break up any emulsions that develop.

g. Transfer organic (top) layer to a 12x75 mm culture tube.

h. Add 0.1 mL of 4:1 methanol:hydrochloric acid and vortex briefly.

i. Evaporate the hexane to dryness under a gentle stream of nitrogen at approximately 40ºC.

j. Reconstitute the dried residue in 0.1 mL of 10:90 methanol:water.

k. Analyze by LC-FTMS using the conditions given below (Sections 10.1 and 10.2).

10 Instrumental Conditions

Appendix 2 contains a checklist of method parameters that should be used to verify proper instrumental conditions prior to analysis of case samples.

10.1 Liquid Chromatograph Parameters (Shimadzu Prominence, or equivalent)

Mobile Phase Compositions	Flow Parameters			Column Parameters	
B: 0.1% formic acid in acetonitrile	total flow	0.3 mL/min		type	C18
	time (min)	%B	%C	length	15 cm
C: 0.1% formic acid in water	0	7.5	92.5	internal diameter	2.1 mm
	5	7.5	92.5	particle size	5 µm
	20	60	40	temperature	40°C
	23	60	40	guard length	7.5 mm
	28	7.5	92.5	guard ID	2.1 mm
	32	7.5	92.5		
	total time	32 min			

10.2 Mass Spectrometer Parameters Using FTMS (Thermo Orbitrap, or equivalent)

Source Parameters		
Mode: Electrospray	Spray Voltage: +5 kV	Capillary Temperature: 250°C
Sheath Gas: 25 (arb units)	Aux Gas: 10 (arb units)	Sweep Gas: 0 (arb units)
All other source parameters are set through the tuning process. See the appropriate IOSS standard operating procedure for details.		
Scan Range	100-350 m/z	
Resolution	30000	

10.3 Cryogrinder (Freezer/Mill) Parameters

Cycles	1
Precool	9 min
Run time	8 min
Cool time	1 min
Rate	10 cps

11 Decision Criteria

The following criteria are used as guidelines in determining the acceptability of the data produced in this assay. In general, compound identification should be based on a comparison of the chromatography and mass spectrometry for the analyte peak of interest with data from a contemporaneously analyzed reference standard, calibrator, or extracted Positive Control.

11.1 Chromatography

The peak of interest should show good chromatographic fidelity, with reasonable peak shape, width, and resolution. In order to be determined acceptable, a chromatographic peak in an unknown sample should compare favorably to a chromatographic peak of the same analyte in a known sample analyzed on the same system in the same or subsequent analytical runs. Additionally, the following two criteria should be met.

11.1.1 Retention Time

The retention time of the peak should be within ± 5% of the retention time (relative or absolute, as appropriate) obtained from injection of a reference standard, calibrator, or Positive Control.

11.1.2 Signal-to-Noise

To justify the existence of a peak, its baseline signal to peak-to-peak noise ratio should exceed 3. Further, the baseline signal for the peak of interest should be at least 10 fold greater than that for any observed peak at similar retention time in a Negative Control or blank injected just prior to the sample.

11.2 Mass Spectrometry

The M+1 for the compound of interest should agree with the theoretical exact mass within 0.003 amu. See Table 4 below for theoretical exact masses.

Table 4: Theoretical Exact Masses (M+1)

Compound Name	Exact Mass (M+1)
Amphetamine	136.112
Methamphetamine	150.128
Ephedrine	166.123
MDA	180.102
MDMA	194.118
MDEA	208.133

11.3 Wash Decision Criteria

If the final wash contains greater than one tenth the amount of an SMA in a given hair sample, the sample's exterior is considered to be possibly contaminated, and will be reported as such.

12 Calculations

See the *Guidelines for Toxicological Quantitations* standard operating procedure (Tox 101) for acceptable practices in calculating quantitative results.

For wash calculations, the total amount of an SMA may be calculated against a new curve, or against the hair curve for that sample.

Example calculations for wash decision criteria:

> Assume 25.0 mg of sample Q1 are washed per the SOP, and that the sample is determined to contain 500 pg methamphetamine per milliliter of hair. Then, the hair sample contained 12500 pg total of methamphetamine (25 x 500). The final wash must contain more than 1250 pg methamphetamine (12500/10) for the Q1 sample to be reported as possibly contaminated.

13 Uncertainty of Measurement

The critical sources of measurement uncertainty in this procedure include:

- historical random uncertainty of repeated measurements
- accuracy of the balance used to deliver the sample
- accuracy of the pipette used to deliver the calibrators
- uncertainty in the concentration of the calibration standards
- precision of the delivery of internal standard

When quantitative results are included in an FBI Laboratory report, the measurement uncertainty will be estimated and reported following the *Chemistry Unit Procedures for Estimating Uncertainty in Reported Quantitative Measurements* standard operating procedure (CUQA 13). Information used to derive uncertainty measurements will be tracked in an electronic database.

14 Limitations

a. Method Performance Parameters:

Compound	LOD (pg/mg)	LLOQ (pg/mg)	Linear Range (pg/mg)	Accuracy (% bias at low and high controls)	Precision (% intermediate at low and high controls)
Amphetamine	21	25	25-1000	+3.81, +22.61	17.63, 17.54
Methamphetamine	14	25	25-1000	+1.72, +6.48	7.77, 8.79
(pseudo)Ephedrine	25	25	25-1000	-0.52, +9.99	11.76, 14.85
MDMA	14	25	25-1000	+5.66, +10.78	7.12, 5.39
MDEA	8	25	25-1000	-2.74, +5.33	9.14, 5.32
MDA	25	N/A; Qual Only			

b. Interferences: None known.

15 Safety

Take standard precautions for the handling of chemicals and biological materials. Refer to the *FBI Laboratory Safety Manual* for guidance.

16 References

Sadeghipour, F. and Veuthey, J., *Journal of Chromatography A*, v. 787 (1997), pp. 137-143

Baselt, R.C., *Disposition of Toxic Drugs and Chemicals in Man*, 7th ed., Biomedical Publications: Foster City, California, 2004.

Guidelines for Toxicological Quantitations (Tox 101); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual.

Chemistry Unit Procedures for Estimating Uncertainty in Reported Quantitative Measurements (CUQA 13); FBI Laboratory Chemistry Unit Quality Assurance and Operations Manual.

Preparation of Chemical Reagents (Tox 103); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual.

Guidelines for Comparison of Mass Spectra (Tox 104); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual.

FBI Laboratory Chemistry Unit – Instrument Operation and Support Subunit SOP Manual.

FBI Laboratory Safety Manual.

Rev.#	Issue Date	History
0	03/08/12	New document. SMA hair analysis was pulled out of Tox 420 into this free standing document.

Approval

Redacted - Signatures on File

Appendix 1: Abbreviated version of the SMA procedure for bench use. (Page 1 of 2)

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Appendix 1: Abbreviated version of the SMA procedure for bench use. (Page 2 of 2)

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Appendix 2: Instrumentation parameters checklist for the SMA procedure.

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